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Development of in vitro propagation system for *Atriplex halimus* L.

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Summary: Explants excised from adult shrubs were surface sterilized and cultured on Murashige and Skoog (MS) basal medium in the presence of plant growth regulators (PGRs) at different concentrations. A high multiplication rate of 7.2-fold was achieved every four weeks on MS medium supplemented with 4.44 µM BA, 0.49 µM IBA and 0.58 µM GA_3. Rooting was achieved with 73% efficiency within 2-4 weeks on agar-gelled MS basal medium free of PGRs. Rooted plantlets were gradually acclimatized to field conditions over 5-6 weeks with 65% efficiency. For in vitro selection for salt tolerance, MS medium was supplemented with increasing concentrations of NaCl ranging between 25 and 1000 mM. This study has demonstrated that in vitro shoots could tolerate up to 600 mM NaCl with optimal growth at 200 mM, while higher concentrations of NaCl affected growth negatively. Growth and shoot number decreased with increasing NaCl concentration with all plantlets died at 1000 mM NaCl.

Keywords: cytokinin, *in vitro* regeneration, shoot formation, Salt tolerance in vitro

Abbreviations: **2,4-D**, 2,4-dichlorophenoxy acetic acid; **2iP**, isopentenyl- adenine; **BA**, N^6-benzyladenine; **GA_3**, gibberellic acid; **ha**, hectare; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **MS**, Murashige and Skoog; **NAA**, αnaphthalene acetic acid; **NN**, Nitsch and Nitsch; **PGR**, plant growth regulator; **WPM**, medium, McCown Woody Plant Medium (1980)

Introduction

Syrian Arab Republic is located on the Eastern coast of the Mediterranean Sea with an area of 18.517.071 ha and a population of about 20.376 million (Anon 2009). Dry land in Syria accounts for about 10 million ha, about 55% of the entire country, with annual rainfall of <200 mm per year for the last 20 years (Wardeh 2005). Desertification is threatening large areas in Syria mainly due to salinity, which has expanded to the irrigated areas in the Eastern part of the country (Harba 2006). Therefore, it is crucial to prevent salinity and desertification in any way possible. One way to rehabilitate and reclaim these soils is by planting salt-tolerant forage species. Among these species is the genus Atriplex, which has recently raised interest where some 100.000 ha have been planted in the Mediterranean basin, Species of the genus Atriplex are known for their high tolerance to aridity and salinity (Le Houerou 1992). Using halophytes to produce forage in saline lands is the best economic solution (Khan and Duke 2001). Halophytes can play a role in soil reclamation in degraded lands (Le Houerou 1992). Atriplex are the dominant plants in arid and semi-arid lands of the world, especially when soil is combined with salinity (Ortíz et al. 2005). Atriplex halimus L. is a member of the Chenopodiaceae and one of the major species used in combating desertification due to its salt tolerance, which is defined as a plant's ability to grow under saline stress (Munns 2002). A. halimus is an important Mediterranean xerohalophyte salt bush species highly resistant to drought (Le Houerou 2000), salinity (Bajji et al. 1998) and heavy metal stress (Lutts et al. 2004). Therefore, this study was carried out to develp an efficient micropropagation system for large-scale rapid clonal multiplication and spreading its cultivation to contribute in alleviating desertification impact on the environment and Humans. Another objective was the selection of in vitro of highly salt-tolerant clones by increasing concentrations of NaCl on *in vitro* grown plantlets.

Materials and methods

Plant material and media. Plant material used in this study was obtained from a shrub of A. halimus grown in a desert area at Altalila Reservation near Palmyra, Syria.

Half-strength Murashige and Skoog (MS, 1962) was used as basal medium for shoot initiation, used throughout this research, supplemented with 3% sucrose (HiMedia-laboratories Pvt. Limited, India) and solidified with 0.7%agar (HiMedia -laboratories Pvt. Limited, India). And for multiplication medium six combinations of hormonal were added to the basic medium **(Table 1).** Three explants were cultured in each glass jar (200 ml) containing 30 ml medium. For rooting medium MS basal medium free of PGRs (as control), 3% sucrose and solidified with 0.7%agar or supplemented with IBA at 0.49 or 2.45 µM. were used. and for salt tolerance MS basal medium was supplemented with increasing concentrations of NaCl (25, 50, 100, 150, 200, 300, 400, 500, 600, 1000 mM) to study the effect of salt on multiplication and selection *in vitro* of shoots which tolerate high concentrations of salt.

Culture conditions. The shoot cultures were maintained in a growth room at 25 ±1°C and a 16-h photo period provided by Philips fluorescent lamps giving an average light intensity of *ca*. 50 µmol $m^{-2}s^{-1}$ photon flux at the surface of culture vessels to assess shoot induction responses. All media were adjusted to pH 5.7 with 1 N KOH or 1 N HCl prior to autoclaving at 121°C, 1.4 kg/cm^2 for 20 min.

Acclimatization. Rooted plantlets were then acclimatized gradually to field conditions by transplanting into pots with a mixture of 2: 1 (v/v) peatmoss : perlite (local origin Mahran, Syria) and were covered with plastic bags and acclimatized gradually to field conditions for a 4-week period.

Experimental design and statistical analysis. Data of all experiments were recorded after 4 weeks from culture on the experimental media. Thirty replicates were used per proliferation treatment and 20 replicates per rooting treatment. Each culture jar was a repetition in a randomized block experimental design, in which the six different media were compared. For each treatment, 30 explants were used with 3explants/jar and 10 replications. Significance was determined at P =0.05 according to Duncan's multiple range test by analysis of variance (ANOVA-2) using the statistical evaluation program SPSS v. 15 .All experiments were repeated three times.

Results and discussion

Disinfection and culture initiation. *In vitro* contamination is a serious problem with tissue culture initiation. Commercial Clorox (5.25%sodium hypochlorite) efficiently surface-disinfected explants. immersion in 15% commercial Clorox for 25 min was the best method for surface disinfection with 79% efficiency decontamination of which 92% were survived (**Fig. 1**). Higher concentrations were too strong, caused bleaching with resulting death of explants. Kenny and Caligari (1996) used 5%commercial Cloroxto surface sterilize *A. glauca* flower clusters. In our study, in contrast, we used 15% Clorox because *A.halimus* is a woody plant, unlike *A.glauca*. In addition, we used axillary buds which are harder than flowers. Mei *et al*. (1997) used 100% commercial bleach for 20 min to sterilize Axillary buds and leaf disc of *A.canescens*.

Effects of PGRs on shoot organogenesis and shoot multiplication *in vitro A. halimus*

Adventitious shoots originating along the cut base of explants and were clearly visible after 3-4 weeks' culture, best shoot multiplication was achieved on media supplemented with 4.44 µM BA, 0.49 µM IBA and 0.58 GA_3 with a multiplication rate of 7.2 shoots/ explants (**Table 1, Fig. 2**) . There was a significant reduction in shoot multiplication rate when BA was replaced with Kin., which resulted in a multiplication rate of 2.1.

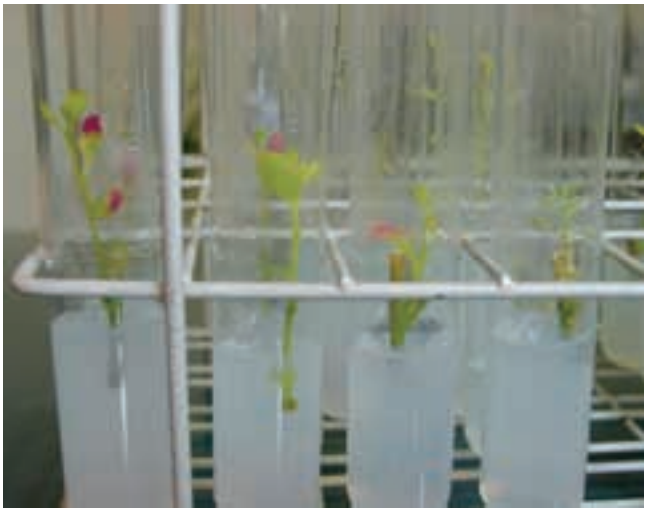

Fig. 1: Initial culture of *Atriplex halimus* L.

Fig. 2: Effect of best combination of growth regulators on in vitro proliferation of *Atriplex halimus* L.

Results presented in **Table 1** demonstrate the effects of a combination of PGRs at different concentrations on the number and length of shoots. The best multiplication rate was achieved on medium containing 4.44 µM BA + 0.49 µM IBA + 0.58 µM GA_3 with an average of 7.26 new shoots/explant every 4 weeks (**Fig.2**). On other hand, control medium gave the best average shoot length (2.08 cm) (**Fig.3**).

The results presented here confirm earlier observations of Malan (2000) who reported that the best medium for shoot multiplication and elongation of *A.canescens* was MS with 0.20 mg/l BA +1 mg/l NAA while MS medium with 0.05 mg/l

BA +0.05 mg/l NAA was best for elongation. Our results also agree with those of Mei *et al*.(1997) who produced shoots from both leaf discs and axillary buds. In their study, the best medium for shoot production was 2 mg/l BA and 0.01 mg/l NAA while the best medium for shoot elongation was PGR-free MS: the number of shoots generated ranged from 0.7 to 9.1/ explant. Reddy *et al.* (1996) found that MS medium with 0.5 mg/l BA or 0.5 mg/l Kin was best for micropropagation of *A. nummularia*.

Table 1 Effect of PGRs combinations on number and length of shoots produced *in vit*ro in *A. halimus* L. within 4 weeks of culture

Treat-ment	Combination of PGRs (Conc. in µM)	Average of shoot number (± SE)*	Average of shoot length (cm) (± SE)*
1	MS + 2.22 BA + 0.49 IBA + 0.58 GA3	4.73 ± 0.15 b	1.05 ± 1.25 b
2	MS + 4.44 BA + 0.49 IBA + 0.58 GA3	7.26 ± 0.32 a	1.0 ± 2.05 b
3	MS + 2.22 Kin + 0.49 IBA + 0.58 GA3	2.96 ± 0.16 bc	1.07 ± 0.61 b
4	MS + 4.44 Kin + 0.49 IBA + 0.58 GA3	2.13 ± 0.27 c	1.06 ± 0.28 b
5	MS + 0.49 IBA + 0.58 GA3	2.36 ± 0.32 c	1.16± 0.55 b
6	MS medium Control (Free of PGRs)	4.4 ± 0.89 bc	2.08 ± 1.22 a
LSD 0.05		2.53	0.59

Different letters within a column indicate significant differences according to Duncan's multiple range test ($P < 0.05$).
Data are means of 30 replications

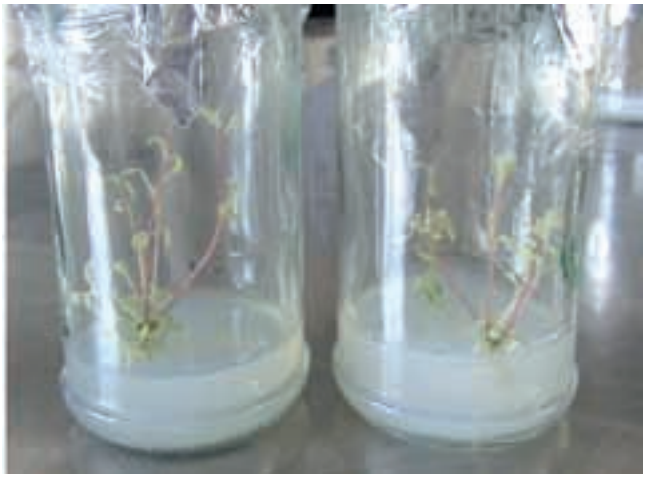

Fig. 3. *In vitro* elongation of *Atriplex halimus* L on control medium

Cytokinins such as TDZ and BAP have considerable effects in inducing regeneration in most woody plants (Korban *et al.*1992; De Bondt *et al.* 1996). BA has been the most commonly used cytokinin for proliferation of many plants (Murai *et al.* 1997). A high concentration of cytokinin with low concentrations of auxins results in a high proliferation efficiency in many plant species (Pierik 1987).

In the present study, however, although induction of shoots was observed in most media tested, BA proved to be more efficient than Kin in shoot induction (**Table 1**).

Our results demonstrate that an optimum combination of PGRs plays a key role in the successful induction of shoot organogenesis *in vitro*. The number of shoots/explant was influenced by the type and concentrations of PGRs used. The number of newly produced shoots varied between 2.1 and 7.2 (**Table 1**). No abnormality, necrosis or chlorosis was observed during culture. Most explants produced shoots and green shoot meristems were seen on a range of media containing BA or Kin and also on control medium free of PGRs.

Multiple shoot induction rate and organogenic response varied significantly to a greater extent according to the explant type and concentrations of PGRs used. Kenny and Caligari (1996) recorded that NN medium was more appropriate than MS medium in their study on *A.glauca*, The IAA/2iP combination at 0.1/0.1 mg/l was better than IAA/Kin at 0.1/0.1mg/l and gave the best response with this species. Zohra *et al.*(2008) found that MS medium with Kin. and 2 mg/l 2,4-D was best for *in vitro* shoot induction from the calli of *A.halimus* and *A.canescens*. However, our study showed that shoots could be induced in only 4weeks unlike the study of Mei *et al.*(1997) who noted that shoot induction in *A.canescens* took at least 2 months. In our study, however, shoot formation could be developed without an intervening callus phase. Moreover, the type of explant and culture medium with specific PGR concentrations influenced organogenesis considerably. We demonstrated that shoot tips and nodal explants can be used for clonal propagation on optimum culture medium.

***In vitro* Rooting**. Proliferated shoot tips (2-3 cm length) were excised and rooted readily on MS medium. Rooting was observed from the cut ends of the shoots within 30 days. All of the developing roots were physically vigorous and healthy. Results in **Table 2** present rooting data: best rooting rate (73.33%) with average number of roots = 3.3 and best average of stem length of 1.63 cm were achieved on MS medium (**Fig. 4**) while 46% rooting efficiency could be recorded onmedium containing 0.49 µM IBA. Our study agrees with the findings of Malan (2000) who noted that MS basal medium without any PGRs was the best medium for rooting. Al-Wasel (1998),who studied the micropropagation of *A. nummularia*, noted that ½-MS without PGRs with phloroglucinol (162 mg/l) was the best rooting media. In contrast, Mei *et al*.(1997) produced *A.canescens* roots using 0.5 mg IBA and 0.1 mg/l GA_3in MS medium with 65% success. In several species, the utilization of high concentrations of IBA produces callus and abnormal roots that affect the survival of explants during acclimatization (Welander 1983; Yepes and Aldwinckle 1994). In the current study, high concentrations of auxins did not give good results and callus formation was induced at the bases of

shoots. Best rooting percentage was better on medium free of PGRs or containing low concentration of auxin (**Table 2, Fig. 4**). Amato *et al.*(1990) recorded best rooting percentage of 67% using IBA at 2000 mg/l although increasing IBA concentration to 4000 mg/l resulted in decreased rooting to 40%. However, in our study, 73% rooting efficiency could be obtained on media without any auxin while use of IBA at 0.49 and 2.45 µM (equiv. 0.1 and 0.5 mg/l) decreased rooting efficiency to 46.6 and 20%, respectively.

Shoot tips and axillary buds from 2-year-old shrubs were excised and used for micropropagation because some studies mentioned that shoot age influences rooting positively probably because of the greater quantity of reserve material, or because of bud dormancy (Amato *et al.* 1990).

Fig. 4. Rooting *in vitro of Atriplex halimus*

Table 2. Effect of IBA concentration on rooting parameters *in vit*ro in *A. halimus* L. within 4 weeks of culture on rooting media

Treat-ment	Concentration of IBA (µM)	Rooting% (±SEM)*	Average of root number (± SE)*	Average of root length (cm) (± SE)*
1	0.49	46.67 ± 0.15 b	1.93 ± 0.60 a	1.14 ± 0.09 a
2	2.45	20 ± 0.1 c	2.33 ± 0.57 a	0.64 ± 0.16 b
3	Control (free of PGRs)	73.33 ± 0.251 a	3.33 ± 1.02 a	1.63± 0.87 a
LSD 0.05		12.56	1. 4	0.49

Different letters within a column indicate significant differences according to Duncan's multiple range test ($P < 0.05$).
Data are means of 20 replications

Acclimatization of rooted plantlets

During *in vitro* culture, plantlets grow under very special conditions, these conditions result in the formation of plantlets of abnormal morphology, anatomy and physiology. After transfer from the *in vitro* to the *ex vitro* conditions, the plantlets have to correct the above-mentioned abnormalities. However, plantlets need gradual changes in environmental conditions to avoid desiccation losses and photo inhibition (Kozai *et al*.1991).Al-Wasel (1998) reported a 55%acclimatization efficiency form icropropagated *A. nummularia*, which is in agreement with the findings of our study but 65% efficiency for *A. halimus* in which rooted plantlets were acclimatized to ambient conditions within 2 months and were later established under greenhouse conditions (**Fig. 5**) and finally in the field under natural conditions (**Fig. 6**). Mei *et al*. (1997) also reported 65% survival of acclimatized shoots of *A.canescens*. Rooted *A.canescens* plantlets were also successfully acclimatized by Malan (2000) but without any reference to the acclimatization efficiency obtained.

Fig. 5. *In vitro* propagated acclimatized *Atriplex halimus* (2-3 months old) grown in the green house

Fig. 6. *In vitro* propagated *Atriplex halimus* (1 year old) under field condition

In vitro selection for salt tolerance in *A. Halimus*

Results of the present study indicate that *A. halimus* is a salt-tolerant species also *in vitro* conditions, where an increase in shoot number could be recorded in media containing up to 200mM NaCl, while higher concentrations of NaCl (400-500-600 mM) inhibited plant growth *in vitro*

(**Table 3**). This result is in agreement with Khan *et al.*(2000) who recorded little inhibition in seedling growth of *A. griffithi* in media containing up to 180mM NaCl, while 360 mM NaCl inhibited plant growth.

Table 3. Effect of NaCl concentrations on the number and length of shoots produced *in vit*ro in *A. halimus* L. within 4 weeks of culture

Medium No.	NaCl conc. (mM)	Mean Shoots Length (cm) (± SE)*	Mean Shoots No. (± SE)*
1	25	3.22 ± 0.56 a	2.76 ± 1.1 abc
2	50	2.17 ± 0.31 bcd	2.6 ± 1.7 abc
3	100	2.76 ± 0.42 ab	3.3 ± 0.75 ab
4	150	2.63 ± 0.40 abc	2.53 ± 0.2 abc
5	200	1.99 ± 0.19 cd	3.73 ± 1.01a
6	300	2.07 ± 0.22 d	2.33 ± 0.15 abc
7	400	1.94 ± 0.22 d	3.1 ± 0.43 abc
8	500	1.57 ± 0.46 d	1.53 ± 0.37 bc
9	600	1.83 ± 0.16 d	1.46 ± 0.46 d
LSD 0.05		0.75	0.16

Different letters within a column indicate significant differences according to Duncan's multiple range test ($P < 0.05$).
Data are means of 30 replications

Our study indicated that *A. halimus* is a highly salt-tolerant species that survived at 600 mM NaCl (salinity of seawater). NaCl at 200 mM was most conducive to plant growth, where the size of leaves was bigger than the control (**Fig.7**). NaCl has been used in most studies on salt tolerance in many plants. However, Nedjimi *et al.* (2006) used $CaCl_2$ to study salt tolerance in *A.halimus* and found that 8 g l^{-1} $CaCl_2$ (about 72 mM) allowed best plant growth. Similar results were found on a study on *A.nummularia* which showed that the growth gain of plants was stimulated by 22% with NaCl at 300 mM, and then reduced significantly in the highest NaCl treatment of 600 mM NaCl with a 26% decrease in growth relative to the control. *A.nummularia* takes up Na^+ and Cl^- in high amounts, but seems to store them efficiently up to an external 300 mM NaCl level only. Above this concentration, however, such a mechanism fails and growth of shoot tissues is negatively affected (Araújo *et al.* 2006). Our results also similar to those of Greenway *et al*.(1966) who reported that growth of *A. nummularia* was optimal at 100 to 200 mM NaCl. Our results also confirm the observations of Ashby and Beadle (1975) that showed that growth of *A. inflata* and *A.nummularia* was greater with NaCl at 600mM than in controls.

The growth of halophytes such as *Atriplex* spp. is stimulated by NaCl concentration which would inhibit the growth of non-halophytes (Osmond *et al.* 1980). It seems that growth response at moderate salinities might be largely the consequence of increased through put of solutes required to derive cell expansion (Khan *et al*. 2001). An important finding is that plants could survive at600 mM NaCl, which might be suitable concentration to use for selection of highly salt-tolerant plants. Higher stress with NaCl induced death in almost all shoots, while just a few could survive, which were selected and multiplied under the same stress conditions for further studies in the near future. Many studies have shown that *Atriplex* spp. such as *A. nummularia*, *A. griffithii* and *A. hortensis* could survive under highly saline conditions, with optimal growth occurring at 5 to 10 g l^{-1}NaCl (85.5-171mM) (Khan *et al.*2000; Wilson *et al*. 2000;Ramos *et al.*2004) which are in agreement with Pribe and Jäger (1978) who found that *Atriplex* spp. vary in their degree of salt tolerance, but all three species studied (*A. halimus*, *A.calotheca*, *A.nilens*) were able to survive at 750 mM NaCl. Storey and Wyn Jones (1979) determined that *A. spongiosa* was able to grow in medium containing 600 mM NaCl with dry mass production decreasing by 50% at 800 mM NaCl. Our study indicates that increasing NaCl concentrations had a negative effect on plant growth; there are many studies indicating that Na^+ and Cl^- content in shoots and roots increased with increasing salinity (Greenway *et al.* 1966; Khan *et al.*2000;Nedjimi*et al.* 2006). Many of the deleterious effects of Na^+ seem to be related to structural and functional integrity of membranes (Kurth *et al*.1986). At high salinities, growth reduction might be caused by a reduced ability to make osmotic adjustment as a result of saturation of the solute uptake system (Munns2002). Expose to saline concentrations (400-600 mM NaCl on *Arthrochnemumm acrostachyum*) has been shown to increase tissue water content of halophytes (Khan *et al.*2005). The water potential of *A. halimus* decreased as salinity levels increase(72-108 mM $CaCl_2$) (Nedjimi *et al.* 2006). Also, the water potential of *A. griffithii* became increasingly negative as media salinity increase (up to 360 mM NaCl) (Khan *et al.*2000). Deleterious effects of salinity are thought to result from low water potentials, ion toxicities, nutrient deficiencies, or a combination of these factors (Munns2002). Growth and survival of halophytes is dependent on high levels of ion accumulation in their tissues for the maintenance of tugor and osmotic adjustment (Flowers *et al*.1977). Halophytes are distinguished by their capacity to produce high concentrations of combatable osmotics to tolerate salinity by increasing ion accumulation (Khan *et al.*2000). Salt tolerance in *A. halimus* could involve a delicate balance among ion accumulation, osmotic adjustment, and maintenance of pressure potential and growth. At relatively high salinities, a significant reduction in growth occurs because of a plant's inability to make an osmotic adjustment, and specific ion toxicities can cause a significant reduction in growth (Nedjimi *et al.*2006).

In the current study we noticed that the plantlet leaves under high NaCl concentration turned whitish in comparison to the control. This can be ascribed to the fact that the shoots of *A.halimus* expunged excess amounts of NaCl to the outer surfaces of leaves giving them this whitish color. Many *Atriplex* species including *A. halimus* have glands on the surface of leaves where salt crystallizes without being harmful (Taiz and Zeiger 1991). Although high amounts of Na^+ accumulate outside *A. halimus* leaves in vesiculated hairs (avoidance structure) (Araújo *et al.* 2006). this protective mechanism does not alone explain the stimulation of growth

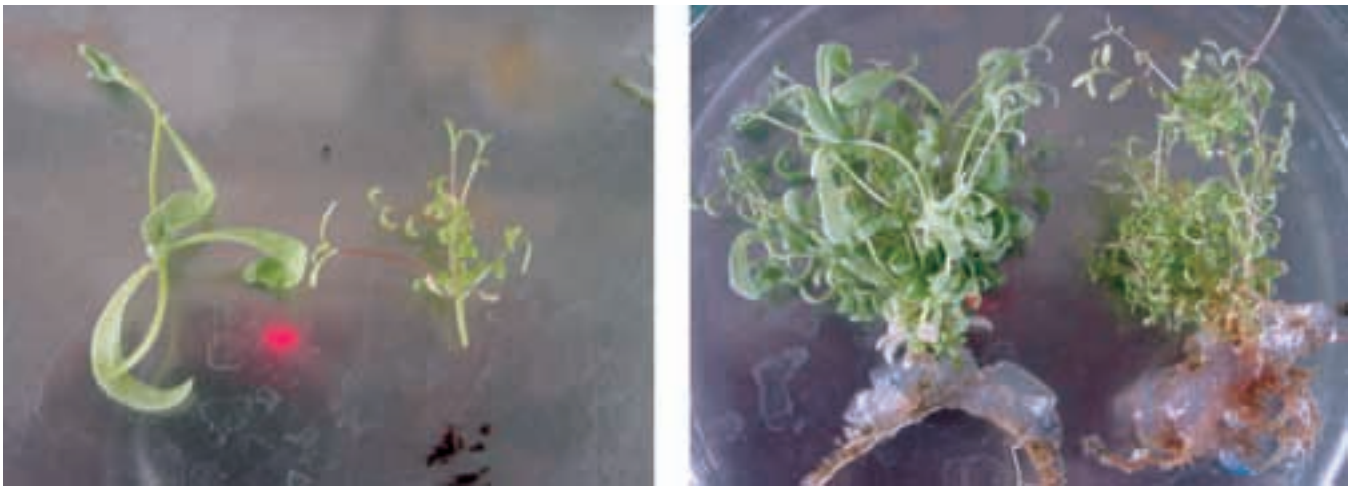

Fig. 7. Effect of NaCl on the growth and size of the leaves *in vitro* of *A. halimus* L.
Left: shoots grown on medium 5 with 200 mM NaCl. **Right**: Control: shoots grown on medium free of NaCl

recorded in the present study. Such an assumption agrees with that of Bajji *et al*. (1998) who studied *A. halimus* grown in media artificially salinized with NaCl. They reported that the physiological mechanism underlying growth stimulation of *Atriplex* plants is still unknown and that such a genus undoubtedly constitutes one of more convenient subjects for investigating the halophytic properties in the plant kingdom. Although not proven, an assumption exists where increased activity of protein synthesis leads to improved growth and is considered of great biochemical and physiological relevance within a given range of NaCl depending on the plant species(Araújo *et al.* 2006). *A. nummularia* may use the controlled uptake of Na^+ balanced by other ions, especially Cl^-, into a cell to drive water into the plant against low external water potential (Blumwald *et al.* 2000). *A. halimus* adopted two different strategies: it behaved as a salt includer at low salinity and as an excluder at high salinity, and growth stimulated in the range of NaCl from 150 to 300 mM (Bajji *et al.* 1998) which agrees with our present study in which optimal growth occurred at 200 mM.

Our study indicates that salinity affected rooting negatively; the higher the salinity, the fewer the roots, which is unlike what Araújo *et al.*(2006) found, i.e. that salinity did not affect rooting. Just a few clones which tolerated high concentrations of NaCl were selected and are being propagated under the same stress conditions for the next step of our study for identification and isolation of salt tolerance gene(s) from these high salt-tolerant clones selected *in vitro* under high stress concentration of NaCl to be used for transformation of crops for salt and drought stress tolerance.

Conclusions

An efficient system for clonal multiplication and shoot organogenesis was developed from cultured shoot tips, nodal explants and axillary buds which can be used for large-scale multiplication to contribute in alleviating desertification impact on the environment and Humans.

The current study demonstrates that thousands of healthy plantlets of this important forage species *A. halimus* can be successfully produced within a short period time.

This highly salt-tolerant halophyte could survive at high salinity concentrations, which makes it available for distribution to be cultivated in arid and semi-arid zones of Syria and other neighbouring countries, where a major problem is insufficiency and irregularity of fodder resources. This also can contribute to combat desertification because of its ability to complete its life cycle under very high saline conditions.

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